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The nature of leukocytic response to mouse mammary tumor implants in C3H/HeJ mice with and without anticoagulation

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THE NATURE OF LEUKOCYTIC RESPONSE TO MOUSE MAMMARY TUMOR IMPLANTS IN C3H/HeJ MICE WITH AND WITHOUT ANTICOAGULATION

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Dated November 20, 1984

DEDICATION

I dedicate this thesis

to

my wife, Cherry, my sons, Michael and Scott,

and my parents,

who have supported and encouraged me, and

to

God who provided the strength to endure.

1984

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INTRODUCTION

Cancer is the second leading cause of death in the United States. The risk of cancer development and subsequent death from the disease increases sharply in the population over 55 years of age(1). Coagulation problems also increase with age and have been implicated in increasing the metastatic spread of cancer. Although cancer treatment has improved constantly it still remains quite toxic and improvements are needed.

Heart disease, cancer, and cerebrovascular disease rank respectively one, two, and three as the leading causes of death in the United States. There are over 729,510 deaths per year from heart disease, approximately 440,000 deaths per year from cancer, and over 175,629 deaths per year from cerebrovascular disease(1).

Approximately 855,000 new cases of cancer are diagnosed each year along with the above mentioned 440,000 deaths. Over 83% of cancer deaths occur in patients over 55 years of age. In all cases the five year survival is less than or equal to 50% in patients with metastatic disease(1).

Arterial and venous thromboembolism contribute to many of these leading causes of death(2), with anticoagulant therapy used routinely in prophylactic and supportive treatment. Evidence supporting the efficacy of anticoagulants in the treatment of certain malignancies is

growing in animal(3,4) and human(5-8) studies. Anticoagulants (e.g. heparin and warfarin), platelet-inhibitory agents (e.g. aspirin, dextran, and dipyridamole), and fibrinolytic agents (e.g. urokinase and streptokinase) have all been reported to inhibit tumor growth. In contrast, conditions which increase coagulation enhance metastasis formation(7).

The concept of metastasis implies that a cancer cell must separate from a primary lesion, move to a distant location, and attach to vascular endothelium. In animal studies, many investigators have pointed to the common association between arrested tumor emboli, fibrin-containing thrombi and platelets; and they have stressed the role of these interactions in precipitating the attachment of circulating malignant cells(9). To prevent the process of hematogenous metastasis formation, inhibition must occur at one of these four levels: 1) at the growth of the primary lesion, 2) at the point of invasion of the vessel walls, 3) at the point of release of viable tumor cells, or 4) at the point of entrapment and growth in distant organs. Warfarin inhibits the entrapment of circulating tumor cells presumably through coagulative mechanisms(10).

Once a metastatic cell becomes attached it requires blood vessels from the host in order to grow. This event is triggered by the tumor cells when they release a diffusable chemical substance called tumor angiogenesis factor (TAF). This substance has the capacity to stimulate nearby blood

vessels to send out new capillaries that grow toward the small colony of tumor cells and penetrate it(11).

Dvorak, et. al.(12), in 1979, hypothesized that tumors might induce and modulate a protective fibrin-gel by secreting molecules capable of activating the host's clotting and fibrinolytic systems. Dvorak suggests that this fibrin-gel (cocoon) around the tumor might enable the tumor cells to develop without challenge from the host's immune surveillance system. Four distinct mediator activities were recognized in the tumor experiment with strain 2 guinea pigs. They found that line 10 tumor cells secreted a vascular permeability factor (VPF), a procoagulant (PC), a plasminogen activator (PA), and a macrophage-migration inhibitory factor (MIF), while nonmalignant control cells secreted none of these. There was one exception in that rabbit kidney cells released a PA activity, presumably urokinase.

Many tumors have tumor-specific antigens on their surfaces with the capability of evoking either humoral or cell-mediated immune response from the host(13). With the protective fibrin-gel, these antigens can be masked from the host allowing the tumor to go undetected.

Warfarin depresses the synthesis in the liver of several factors which are known to be active in the coagulation mechanism leading to fibrin formation. By inhibiting fibrin formation, warfarin could lead to increased detection of tumor by the host. If the fibrin-gel

is instrumental in angiogenesis then production of new blood vessels to the tumor would also be inhibited, thus leading to decreased tumor growth and increased tumor death. As an anticoagulant, warfarin would also assist in reducing coagulation problems associated with cardiovascular disease and increased metastasis formation in cancer patients. Since the population over 55 years of age is at the greatest risk in all three categories (i.e. heart disease, cancer, and cerebrovascular disease), it seems logical that patients in this age group could benefit(1,14-16) from anticoagulant therapy. Also, it is not uncommon for these patients to have more than one of these three death-causing diseases, and their gain could be even more significant.

As stated above, studies indicate that anticoagulants may be beneficial in the treatment of certain malignant tumors by inhibiting tumor metastasis. Warfarin as an anticoagulant has been tested and proven safe on long-term administration. The therapeutic dose of warfarin has been well-established, and it is effective orally. Its risk of toxic overdose can be minimized by monitoring the prothrombin time, and its side effects, other than hemorrhage, are rare. If toxic doses of warfarin are given, the effects on the coagulation mechanism can be readily reversed by vitamin K and/or transfusion therapy(7). Therefore, warfarin is a logical drug to use in conducting the study.

Another factor important in disease control is host

immune surveillance. If the fibrin-gel is tumor protective(3,4,12), then removal of the gel should expose the tumor to normal defensive mechanisms. Based on animal studies by Folkman(11) and Santer(17), this tumor exposure to immune surveillance should then produce altered leukocyte activity. Shortly after the start of warfarin therapy, normal immune response should show an increase in neutrophils (PMNs) and macrophages in the area of the tumor. There should also be an increase in T-lymphocytes, B-lymphocytes and null-lymphocytes. B-lymphocytes should then be stimulated to produce antibodies to the exposed tumor surface antigens allowing complement fixation, K cell activity, and tumor death(17).

Nuclear medicine techniques using Indium-111 Oxine now allow labeling of cellular blood components so that immune responses can be observed in humans. Indium-111 Oxine forms a nonpolar lipophilic complex useful for labeling platelets for thrombus detection, and leukocytes (WBCs) for leukocyte kinetic studies. The labeled WBCs can then be used to locate infection, inflammation, or tumorcidal activity(18). Indium-111 Oxine has been reported to have no side effects and to be safe as a diagnostic agent in humans(19).

MATERIALS AND METHODS

Mouse mammary tumor is a common form of cancer used for experimental animal study. This investigation was designed to study the nature of leukocytic response to mouse mammary tumor implants in C3H/HeJ mice with and without anticoagulants. The main thrust of the research was in three areas: first, to develop a background in light microscope morphology of spontaneous mouse mammary tumor and its change with first and second passage into normal mice; second, to analyze change in leukocytic response in sham operated, tumor- and liver-implanted mice; and third, to analyze changes in leukocytic response to tumor implants with and without anticoagulation.

Experiment I: Light Microscope Morphology of Spontaneous and Passaged C3H/HeJ Mouse Mammary Tumors.

Goal: To become familiar with the parenchyma and stroma of spontaneous and passaged C3H/HeJ mouse mammary tumors in order to evaluate morphologic responses to experimental procedures.

Method: Four C3H/HeJ mice with spontaneous mammary tumors were sacrificed using diethyl ether. The dorsal surface of the dead mouse was swabbed with 70% ethanol, an incision made, skin retracted and pinned back, and the tumor

carefully dissected from the surrounding tissue of each mouse. When separated from the surrounding tissue, the tumor was washed several times in sterile isotonic saline. A portion of the tumor was then dissected into pieces weighing approximately 10 mg. (Tumor not required for implantation was fixed in 10% formalin and paraffin slides prepared.) A single segment (approximately 10 mg) was implanted into each of two virgin female C3H/HeJ mice using a 23 gauge trocar. The trocar was introduced subcutaneously into the tissue via a skin incision and directed toward the implantation site midway between the left shoulder and left hip in the axillary line. Tumor implantation was completed by introducing a plunger into the barrel of the trocar. A clockwise, followed by a counter-clockwise, rotation of the plunger insured complete evacuation of the tumor. The trocar and plunger were then carefully removed. No sutures were required to repair the skin incision. Three weeks after the implantation the two mice (first passage) were sacrificed and the tumors removed and prepared in a manner like that described above. A segment of tumor from one mouse was then implanted into one normal mouse and a segment of the second tumor into a second normal mouse. Three weeks later the two mice (second passage) were sacrificed and the tumor removed and fixed in 10% formalin with paraffin slides prepared (Table I).

Table I

EXPERIMENT I

(TUMOR IMPLANT PASSAGE)

FOUR C3H/HeJ MICE WITH SPONTANEOUS MAMMARY TUMORS

DAY 1	ST1	ST2	ST3	ST4
DAY 22	TP1-1	TP2-1	TP3-1	TP4-1
	TP1-1	TP2-1	TP3-1	TP4-1
DAY 43	TP1-2	TP2-2	TP3-2	TP4-2
	TP1-2	TP2-2	TP3-2	TP4-2

(ST1, ST2, ST3, ST4) represents spontaneous tumor mice numbers 1, 2, 3, and 4.

(TP1-1, TP2-1, TP3-1, TP4-1) represents transplant number 1 from each spontaneous tumor mouse number 1, 2, 3, and 4.

(TP1-2, TP2-2, TP3-2, TP4-2) represents transplant number 2 from each spontaneous tumor mouse number 1, 2, 3, and 4.

Experiment II: Leukocytic Response in Sham operated, Tumor- and Liver-implanted C3H/HeJ Mice.

Goal: To characterize the leukocytic response to implanted C3H/HeJ spontaneous tumor segments by contrasting that response with those of syngeneic mice with implants of normal (liver) tissue and mice that were sham operated upon.

Method: To obtain material for implantation, a female donor C3H/HeJ mouse with a spontaneous mammary tumor and a normal mouse were sacrificed using diethyl ether.

The dorsal surface of the dead tumor mouse was swabbed with 70% ethanol, an incision made, skin retracted and pinned back, and the tumor carefully dissected from the surrounding tissue. When separated from surrounding tissue, the tumor was washed several times in sterile isotonic saline. The tumor was then dissected into pieces weighing approximately 10 mg. A single tumor segment (approximately 10 mg) was then implanted into each of 12 virgin female C3H/HeJ mice using a 23 gauge trocar. The trocar was introduced subcutaneously into the tissue via a skin incision and directed toward the implantation site midway between the left shoulder and left hip in the axillary line. Tumor implantation was completed by introducing a plunger into the barrel of the trocar. A clockwise, followed by a counter-clockwise, rotation of the plunger insured complete evacuation of the tumor. The trocar and plunger were carefully removed. No sutures were required to repair the

skin incision.

The anterior surface of the dead normal mouse was swabbed with 70% ethanol, an incision made, skin retracted and pinned back, and the normal liver was carefully removed from the mouse. When separated the liver was then dissected into pieces weighing approximately 10 mg. A second group had a single liver segment (approximately 10 mg) implanted into each of 12 virgin female C3H/HeJ mice using a 23 gauge trocar. The trocar was introduced subcutaneously into the tissue via a skin incision and directed toward the implantation site midway between the left shoulder and left hip in the axillary line. Liver implantation was completed by introducing a plunger into the barrel of the trocar. A clockwise, followed by a counter-clockwise, rotation of the plunger insured complete evacuation of the liver. The trocar and plunger were then carefully removed. No sutures were required to repair the skin incision.

A third group of 12 virgin female C3H/HeJ mice had the empty 23 gauge trocar introduced subcutaneously tissue via a skin incision and directed toward the implantation site midway between the left shoulder and left hip in the axillary line. A plunger was introduced into the barrel of the trocar and clockwise, followed by counter-clockwise, rotation of the plunger was performed. The trocar and plunger were carefully removed. No sutures were required to repair the skin incision.

Three mice from each group were sacrificed on day 1 (24 hours after implant operation), day 2, day 4, and day 8 (Table II).

A posterior full midline incision was made and the skin flap pinned and observed. The tumor, liver, or sham track was removed in block (with skin attached) and fixed in 10% formalin with paraffin slides prepared.

The slides from each group were observed for a change in leukocyte activity around the tumor, liver or sham implant site. Procedures for measuring change are further detailed under method of analysis (p.14).

Experiment III: Effects of Heparin and Warfarin on C3H/HeJ Mouse Mammary Tumor Implant: Leukocytic Response Alterations.

Goal: To describe changes in leukocytic response to tumor implants in control mice versus tumor-bearing mice receiving heparin or warfarin.

Method: To obtain material for implantation, a female donor C3H/HeJ mouse with a spontaneous mammary tumor was sacrificed using diethyl ether. The dorsal surface of the dead mouse was swabbed with 70% ethanol, an incision made, skin retracted and pinned back, and the tumor carefully dissected from the surrounding tissue. When separated from the surrounding tissue, the tumor was washed several times in sterile isotonic saline. The tumor was then dissected

Table II

EXPERIMENT II

SHAM OPERATED, TUMOR- AND LIVER-IMPLANTED C3H/HeJ MICE

(TOTAL OF 36 MICE DIVIDED INTO 3 GROUPS)

	SHAM	TUMOR	LIVER
	12 MICE	12 MICE	12 MICE
DAY 1	3	3	3
DAY 2	3	3	3
DAY 4	3	3	3
DAY 8	3	3	3

into pieces weighing approximately 10 mg. A single tumor segment (approximately 10 mg) was implanted into each of 36 virgin female C3H/HeJ mice using a 23 gauge trocar. The trocar was introduced subcutaneously into the tissue via a skin incision and directed toward the implantation site midway between the left shoulder and left hip along the axillary line. Tumor implantation was completed by introducing a plunger into the barrel of the trocar. A clockwise, followed by a counter-clockwise, rotation of the plunger insured complete evacuation of the tumor. The trocar and plunger were then carefully removed. No sutures were required to repair the skin incision.

The 36 mice were divided into three groups of 12 mice each. Twelve mice in the control group were given 0.01 ml per gram of mouse weight (point body weight concentration) of normal saline (0.9% sodium chloride) injected intraperitoneally (i.p.) once a day started day -3 (day -3 refers to injections started 3 days before the tumors were implanted) and continued through day 14.

Twelve mice were in the warfarin group with 0.01 ml per gram of mouse weight of a point body weight equivalent to 0.75 mg/kg injected i.p. once a day from day -3 and continued through day 14.

Twelve mice were in the heparin group with 0.01 ml per gram of mouse weight of a point body weight concentration equivalent to 0.4 mg/kg injected i.p. twice a day started on day -3 and continued through day 14.

Two mice from each group were sacrificed on days 2, 4, 7, 9, 11 and 14 (Table III).

After sacrifice, a posterior full midline incision was made and the skin flap pinned and observed. The tumor was removed in block and fixed in 10% formalin with paraffin slides prepared.

The slides from each group were analyzed for change in leukocytic response to tumor implants in control mice versus tumor-bearing mice receiving heparin or warfarin.

Method of Analysis: Both experiments II and III were analyzed in the same manner using a Nikon microscope with a Nikon transformer light source. The observations were performed from a microscopic view of 400 power. The border of the tumor was located under the microscope at 40 to 100 power. The interface of the tumor with the infiltrative boundary was then viewed at 400 power. The left 10 power optic of the microscope was equipped with a 65 X 45 mm. rectangular box with a cross-hair dividing the box in half. The box was positioned with the cross-hair dividing the interior (tumor portion of the slide) from the boundary (the infiltrative interface around the tumor). A physical count of the cells in each half of the square was performed. One-half of the total box outlined the area (or field) of the cell count for the interior and the other half of the box outlined the area of the count in the boundary. The cells were counted and classified as polymorphonuclear leukocytes (PMNs), lymphocytes, histiocytes, fibroblasts,

Table III

EXPERIMENT III

TUMOR-IMPLANTED C3H/HeJ MICE WITH AND WITHOUT ANTICOAGULANTS

(TOTAL OF 36 MICE DIVIDED INTO 3 GROUPS)

	CONTROL (SALINE) 12 MICE	HEPARIN 12 MICE	WARFARIN 12 MICE
DAY 2	2	2	2
DAY 4	2	2	2
DAY 7	2	2	2
DAY 9	2	2	2
DAY 11	2	2	2
DAY 14	2	2	2

or tumor cells. The cell counts were then verified by a second person. A total of three separate observations and counts was performed on each tumor block for each mouse.

The numbers generated from the cell counts in the boundary ,and the interior were then combined into groups. In Experiment II one group was made up of those mice sacrificed from day 1 through day 4 and the second group was represented by those mice sacrificed on day 8. In Experiment III the two groups consisted of mice sacrificed on days 2 through 4 and mice sacrificed on days 7 through 14. These data were then statistically evaluated using the Dunnett's-t test for two or more groups and comparisons were made to normal immune responses in the control groups.

RESULTS

Table IV of Experiment I represents a list of the data obtained from microscopic observations at 400 power. These data were not statistically evaluated since the experiment was conducted for the purpose of familiarization for the investigator.

Statistical Comparisons

Tables V through XIV represent the raw data from Experiment II while Tables XV and XVI report the mean data and corresponding standard error of the mean.

Table XV shows an increase ($P<0.05$) in the number of PMNs in response to tumor-implant when compared to the sham operated group. Lymphocyte activity is also increased ($P<0.01$) for both liver and tumor implants when compared to the sham operated group. Histiocyte response was increased ($P<0.05$) in the liver-implanted group when compared to the sham operated group. A highly significant difference in the number of fibroblasts ($P<0.01$) was observed around the boundary in the day 1 through 4 liver- and tumor-implanted mice. The number of fibroblasts was significantly lower around the tumor and the liver implants.

Table XVI shows an increase ($P<0.05$) in the lymphocytic response on day 8 in the tumor-implanted group when compared to the liver-implanted group. Shown also is a decrease

($P<0.05$) in the number of histiocytes appearing in the interior of the tumor-implanted group between days 1 and 4 when compared to the liver-implanted group.

Table IV

EXPERIMENT I

Raw Data for Light Microscope Morphology of Spontaneous and Passaged C3H/HeJ Mouse Mammary Tumors.

Mouse Number	Day	Glandular Arrangement Present Yes/No	Secretions Present Yes/No	Cytoplasm Present Yes/No
ST1	1	N	N	N
TP1-1	22	N	N	N
TP1-2	43	Y/N	N	N
ST2	1	Y	Y	Y
TP2-1	22	Y/N	N	Y
TP2-2	43	Y/N	N	Y
ST3	1	Y/N	Y	Y
TP3-1	22	N	N	N
TP3-2	43	N	N	N
ST4	1	N	N	N
TP4-1	22	N	N	N
TP4-2	43	N	N	N

(ST1, ST2, ST3, ST4) represents spontaneous tumor mice numbers 1, 2, 3, and 4.

(TP1-1, TP2-1, TP3-1, TP4-1) represents transplant number 1 from each spontaneous tumor mouse number 1, 2, 3, and 4.

(TP1-2, TP2-2, TP3-2, TP4-2) represents transplant number 2 from spontaneous tumor mouse number 1, 2, 3, and 4.

Table V

EXPERIMENT II

Raw Data for Boundary of Sham Operated C3H/HeJ Mice

Sample No.	Day	PMNs	Lymph	Hist	Fib
1	1	9	1	1	5
2	1	4	1	7	16
3	1	1	0	7	15
4	1	0	0	9	0
5	1	0	0	6	11
6	1	0	1	7	4
7	1	5	2	7	3
8	1	5	2	10	1
9	1	4	2	6	4
10	2	9	8	8	0
11	2	0	1	7	14
12	2	0	0	16	15
13	2	3	5	14	0
14	2	0	1	16	7
15	2	1	0	12	0
16	2	0	0	7	0
17	2	2	1	9	5
18	2	0	2	6	5
19	4	0	8	9	0
20	4	0	0	6	7
21	4	0	0	12	2
22	4	3	6	12	0
23	4	0	2	3	8
24	4	0	3	2	5
25	4	0	0	2	12
26	4	0	0	3	4
27	4	0	0	2	7

Table VI

EXPERIMENT II

Raw Data for Boundary of Sham Operated C3H/HeJ Mice

Sample No.	Day	PMNs	Lymph	Hist	Fib
1	8	1	2	3	2
2	8	0	1	9	4
3	8	0	2	11	7
4	8	0	6	15	1
5	8	0	0	12	0
6	8	0	0	12	4
7	8	0	3	9	3
8	8	0	1	10	12
9	8	2	0	9	2

Table VII

EXPERIMENT II

Raw Data for Boundary of Liver-Implanted C3H/HeJ Mice

Sample No.	Days	PMNs	Lymph	Hist	Fib
1	1	15	6	3	0
2	1	14	7	10	0
3	1	11	9	9	0
4	1	11	4	17	1
5	1	18	9	16	0
6	1	12	8	12	0
7	1	0	22	7	0
8	1	3	12	16	0
9	1	3	9	11	0
10	2	6	6	7	0
11	2	5	7	16	0
12	2	9	3	11	0
13	2	5	8	0	0
14	2	2	16	19	0
15	2	8	10	12	2
16	2	7	12	1	0
17	2	3	5	11	0
18	2	3	8	13	0
19	4	2	6	15	0
20	4	1	3	11	2
21	4	2	14	5	0
22	4	0	3	4	0
23	4	1	2	6	1
24	4	0	11	17	2
25	4	1	7	16	5
26	4	2	8	22	3
27	4	1	1	15	2

Table VIII

EXPERIMENT II

Raw Data for Boundary of Liver-Implanted C3H/HeJ Mice

Sample No.	Days	PMNs	Lymph	Hist	Fib
1	8	0	4	22	0
2	8	1	1	12	6
3	8	1	3	30	3
4	8	0	3	10	0
5	8	0	0	10	0
6	8	0	3	7	0
7	8	0	9	8	7
8	8	0	8	28	0
9	8	2	2	15	1

Table IX

EXPERIMENT II

Raw Data for Boundary of Tumor-Implanted C3H/HeJ Mice

Sample No.	Days	PMNs	Lymph	Hist	Fib
1	1	5	9	0	0
2	1	5	6	4	2
3	1	6	8	8	0
4	1	6	2	16	0
5	1	21	10	2	0
6	1	4	19	9	4
7	1	67	16	0	0
8	1	8	12	10	0
9	1	24	11	11	0
10	2	15	4	9	0
11	2	6	1	5	0
12	2	11	4	12	3
13	4	1	3	9	2
14	4	2	5	15	0
15	4	0	2	17	6
16	4	1	6	17	2
17	4	0	9	9	1
18	4	0	4	9	1
19	4	1	1	21	3
20	4	1	9	6	0
21	4	3	12	9	0

Table X

EXPERIMENT II

Raw Data for Boundary of Tumor-Implanted C3H/HeJ Mice

Sample No.	Days	PMNs	Lymph	Hist	Fib
1	8	1	2	7	4
2	8	1	4	13	7
3	8	0	5	14	4
4	8	2	2	8	1
5	8	1	3	17	0
6	8	2	3	15	0
7	8	1	3	5	3
8	8	0	2	12	0
9	8	0	1	14	4

Table XI

EXPERIMENT II

Raw Data for Interior of Liver-Implanted C3H/HeJ Mice

Sample No.	Days	PMNs	Lymph	Hist	Fib
1	1	76	16	0	0
2	1	3	5	35	0
3	1	16	7	41	0
4	1	13	4	21	0
5	1	9	2	25	0
6	1	4	3	19	0
7	1	0	3	7	0
8	1	3	3	21	0
9	1	5	3	25	0
10	2	0	3	0	0
11	2	0	9	30	2
12	2	2	4	13	5
13	2	0	3	0	0
14	2	3	8	11	0
15	2	0	4	11	0
16	2	2	2	7	0
17	2	1	1	5	0
18	2	2	7	8	0
19	4	3	8	8	0
20	4	3	6	11	1
21	4	0	6	10	4
22	4	4	13	17	0
23	4	2	9	18	0
24	4	5	3	12	0
25	4	0	4	8	0
26	4	0	2	15	0
27	4	1	11	20	0

Table XII

EXPERIMENT II

Raw Data for Interior of Liver-Implanted C3H/HeJ Mice

Sample No.	Days	PMNs	Lymph	Hist	Fib
1	8	0	2	1	0
2	8	0	5	13	4
3	8	2	2	26	0
4	8	0	3	11	3
5	8	0	0	10	2
6	8	0	1	10	0
7	8	2	8	29	1
8	8	1	3	30	0
9	8	0	0	39	0

Table XIII

EXPERIMENT II

Raw Data for Interior of Tumor-Implanted C3H/HeJ Mice

Sample No.	Days	PMNs	Lymph	Hist	Fib
1	1	1	5	3	0
2	1	3	10	15	0
3	1	5	15	16	0
4	1	3	7	11	0
5	1	3	6	0	0
6	1	2	6	10	0
7	1	8	15	10	0
8	1	1	4	6	0
9	1	2	5	11	1
10	2	0	5	0	0
11	2	2	2	0	1
12	2	2	5	8	0
13	4	0	3	13	0
14	4	1	6	20	0
15	4	0	3	4	0
16	4	4	12	14	0
17	4	0	1	20	0
18	4	0	2	19	1
19	4	0	3	4	0
20	4	1	7	5	0
21	4	0	3	6	2

Table XIV

EXPERIMENT II

Raw Data for Interior of Tumor-Implanted C3H/HeJ Mice

Sample No.	Days	PMNs	Lymph	Hist	Fib
1	8	4	5	15	1
2	8	0	3	15	1
3	8	1	1	14	4
4	8	4	6	13	2
5	8	1	20	10	1
6	8	4	14	10	3
7	8	0	7	12	0
8	8	0	7	7	0
9	8	0	10	10	0

Table XV

EXPERIMENT II

Mean Values ± 1 Standard Error of the Mean

with Dunnett's-t applied for the Boundary of the

Sham Operated, Liver- and Tumor-Implanted C3H/HeJ Mice

Cell Type	Days	No. of Observarions (S)	(L)	(T)	Sham (S) MEAN ± SEM	Liver (L) MEAN ± SEM t=	Tumor (T) MEAN ± SEM t=	Results p-value ct=
PMNs	1-4	27	27	21	1.7 0.52	5.4 0.99 -1.51	*8.9 3.25 **-2.96	*p<0.05 **2.26
	8	9	9	9	0.3 0.24	0.4 0.24 -0.32	0.9 0.26 -1.59	p>0.05 2.35
Lymphs	1-4	27	27	21	1.7 0.46	***8.0 0.88 **-5.52	***7.3 1.07 **-4.89	***p<0.01 **2.89
	8	9	9	9	1.7 0.65	3.7 1.0 -1.95	2.8 0.40 -1.08	p>0.05 2.35
Hist	1-4	27	27	21	7.6 0.80	*11.2 1.08 **-2.45	9.4 1.22 -1.24	*p<0.05 **2.26
	8	9	9	9	10.0 1.09	15.8 2.91 -2.09	11.7 1.35 -0.60	p>0.05 2.35
Fib	1-4	27	27	21	5.6 1.00	***0.8 0.24 **5.06	***1.1 0.37 **4.67	***p<0.01 **2.89
	8	9	9	9	3.9 1.22	1.9 0.93 1.41	2.6 0.82 0.94	p>0.05 2.35

t = Dunnett's-t value, ct = Critical-t value, (S) = Sham, (L) = Liver, (T) = Tumor, SEM = Standard Error of the Mean.
* p<0.05 and *** p<0.01 = significant value.
** t values > ct representing significant results.

Table XVI

EXPERIMENT II

Mean Values ± 1 Standard Error of the Mean

with Dunnett's-t applied for the Interior of the

Sham Operated, Liver- and Tumor-Implanted C3H/HeJ Mice

Cell Type	Days	No. of Observations (S)	(L)	(T)	Sham (S) Mean ---- ± SEM	Liver (L) Mean ---- ± SEM	Tumor (T) Mean ---- ± SEM t=	Results p-value ct=
PMNs	1-4	27	21			5.8 2.80	1.8 0.45 1.26	p>0.05 2.01
	8	9	9			0.6 0.29	1.6 0.63 -1.45	p>0.05 2.12
Lymph	1-4	27	21			5.5 0.70	6.0 0.87 -0.40	p>0.05 2.01
	8	9	9			2.6 0.82	*8.1 1.95 **-2.63	*p<0.05 **2.12
Hist	1-4	27	21			*14.7 1.98	*9.3 1.41 **2.14	*p<0.05 **2.01
	8	9	9			18.8 4.18	11.8 0.91 1.64	p>0.05 2.12
Fib	1-4	27	21			0.4 0.24	0.2 0.12 0.71	p>0.05 2.01
	8	9	9			1.1 0.51	1.3 0.47 -0.32	p>0.05 2.12

t = Dunnett's-t value, ct = Critical-t value, (S) = Sham, (L) = Liver, (T) = Tumor, SEM = Standard Error of the Mean.
* $p<0.05$ = Significant Value.
** t value > ct represents significant results.

Tables XVII through XXVIII contain the raw data for Experiment III while Tables XXIX and XXX report the mean data and corresponding standard error of the mean.

Experiment III (Table XXIX) shows that the number of fibroblasts seen from day 7 through 14 in the warfarin group was significantly ($P<0.05$) less than in the control (saline injected) group. The number of boundary lymphocytes from day 7 through 14 was increased ($P<0.05$) in both the heparin and warfarin groups when compared to the saline group.

Table XXX shows an increased number of tumor cells ($P<0.01$) in the interior of the warfarin group when compared to the saline group between days 1 and 4.

Table XVII

EXPERIMENT III

Raw Data for Boundary of Tumor-Implanted C3H/HeJ Mice with Saline Injection

Sample No.	Days	PMNs	Lymph	Hist	Fib	Tumor
1	2	5	5	2	2	10
2	2	6	6	5	1	9
3	2	7	10	3	1	4
4	2	22	4	0	3	0
5	2	19	4	3	0	0
6	2	17	4	5	1	0
7	4	8	4	14	3	0
8	4	4	3	8	10	0
9	4	4	4	9	14	0
10	4	4	4	12	0	3
11	4	1	0	16	0	0
12	4	1	1	9	0	0

Table XVIII

EXPERIMENT III

Raw Data for Boundary of Tumor-Implanted C3H/HeJ Mice with Saline Injection

Sample No.	Days	PMNs	Lymph	Hist	Fib	Tumor
1	7	1	0	2	3	0
2	7	0	0	5	0	0
3	7	0	0	6	3	0
4	7	0	1	10	1	0
5	7	0	3	4	0	0
6	7	0	1	6	1	0
7	9	0	0	5	0	2
8	9	0	0	4	0	0
9	9	0	0	4	0	1
10	9	0	1	3	0	1
11	9	0	2	15	9	3
12	9	0	1	1	3	1
13	11	0	0	11	1	0
14	11	1	0	9	5	0
15	11	0	2	5	5	1
16	11	1	0	4	0	0
17	11	1	0	6	0	0
18	11	0	0	4	2	0
19	14	2	0	1	9	0
20	14	1	0	7	9	0
21	14	1	0	29	3	1
22	14	0	0	3	10	0
23	14	1	0	4	13	0
24	14	0	0	9	20	0

Table XIX

EXPERIMENT III

Raw Data for Boundary of Tumor-Implanted C3H/HeJ Mice with Heparin Injection

Sample No.	Days	PMNs	Lymph	Hist	Fib	Tumor
1	2	7	12	1	2	0
2	2	3	2	3	6	1
3	2	12	3	5	1	0
4	2	12	5	3	10	0
5	2	10	8	3	3	0
6	2	2	3	2	0	0
7	4	1	2	7	1	0
8	4	2	1	10	0	0
9	4	4	3	21	5	0
10	4	0	2	4	0	0
11	4	18	1	16	1	0
12	4	6	1	10	4	1

Table XX

EXPERIMENT III

Raw Data for Boundary of Tumor-Implanted C3H/HeJ Mice with Heparin Injection

Sample No.	Days	PMNs	Lymph	Hist	Fib	Tumor
1	7	2	2	12	6	0
2	7	4	7	10	3	4
3	7	0	8	10	3	0
4	9	2	10	12	3	2
5	9	1	1	14	0	1
6	9	4	3	10	9	1
7	9	0	0	4	0	3
8	9	0	0	4	0	1
9	9	0	0	4	0	1
10	11	1	3	13	1	1
11	11	0	3	7	1	3
12	11	0	0	4	0	0
13	11	0	0	0	0	0
14	11	2	1	23	0	0
15	11	1	1	8	4	0
16	14	1	1	12	0	0
17	14	0	1	7	4	0
18	14	0	3	13	7	1

Table XXI

EXPERIMENT III

Raw Data for Boundary of Tumor-Implanted C3H/HeJ Mice with Warfarin Injection

Sample No.	Days	PMNs	Lymph	Hist	Fib	Tumor
1	2	20	3	4	4	2
2	2	14	0	7	5	0
3	2	1	0	1	0	1
4	4	5	2	6	2	0
5	4	10	1	4	0	0
6	4	11	6	12	0	0

Table XXII

EXPERIMENT III

Raw Data for Boundary of Tumor-Implanted C3H/HeJ Mice with Warfarin Injection

Sample No.	Days	PMNs	Lymph	Hist	Fib	Tumor
1	7	0	0	18	3	0
2	7	0	2	8	0	0
3	7	1	4	17	1	0
4	7	0	2	7	0	1
5	7	0	0	13	0	0
6	7	1	0	7	0	0
7	9	0	1	5	1	2
8	9	0	1	5	9	0
9	9	0	3	5	1	0
10	9	1	1	6	3	1
11	9	1	8	13	1	1
12	9	4	0	8	0	0
13	11	1	7	9	2	0
14	11	2	5	16	0	0
15	11	2	11	16	1	0
16	11	0	0	2	0	0
17	11	3	0	1	0	0
18	11	0	0	2	0	0
19	14	1	1	5	2	0
20	14	2	2	16	1	0
21	14	1	2	13	0	0

Table XXIII

EXPERIMENT III

Raw Data for Interior of Tumor-Implanted C3H/HeJ Mice with Saline Injection

Sample No.	Days	PMNs	Lymph	Hist	Fib	Tumor
1	2	0	2	2	0	17
2	2	0	1	2	1	17
3	2	0	1	0	7	0
4	2	1	2	1	0	2
5	2	0	0	0	27	0
6	2	2	1	3	13	6
7	4	6	4	10	5	0
8	4	1	2	11	4	3
9	4	0	2	3	1	0
10	4	7	3	9	0	10
11	4	4	4	10	0	1
12	4	9	5	9	2	3

Table XXIV

EXPERIMENT III

Raw Data for Interior of Tumor-Implanted C3H/HeJ Mice with Saline Injection

Sample No.	Days	PMNs	Lymph	Hist	Fib	Tumor
1	7	0	0	14	14	5
2	7	2	0	22	2	4
3	7	6	0	23	3	3
4	7	1	4	11	5	1
5	7	1	3	21	8	2
6	7	2	6	19	2	0
7	9	0	4	8	7	8
8	9	0	1	18	4	5
9	9	3	1	6	0	10
10	9	5	2	12	5	1
11	9	1	4	14	8	14
12	9	4	3	12	11	6
13	11	0	2	27	5	3
14	11	0	3	13	13	1
15	11	2	3	24	0	6
16	11	1	4	4	8	1
17	11	3	4	2	2	0
18	11	0	3	6	9	0
19	14	5	1	9	2	1
20	14	0	1	11	0	4
21	14	0	3	5	2	3
22	14	2	1	19	8	2
23	14	0	0	23	3	1
24	14	3	5	29	3	1

Table XXV

EXPERIMENT III

Raw Data for Interior of Tumor-Implanted C3H/HeJ Mice with Heparin Injection

Sample No.	Days	PMNs	Lymph	Hist	Fib	Tumor
1	2	0	3	5	0	16
2	2	5	2	4	2	12
3	2	5	2	3	5	8
4	2	4	1	7	5	4
5	2	0	1	7	8	3
6	2	1	0	7	4	7
7	4	4	2	19	2	6
8	4	10	5	13	0	7
9	4	2	1	3	1	1
10	4	0	1	13	0	7
11	4	5	1	7	3	1
12	4	9	1	10	0	2

Table XXVI

EXPERIMENT III

Raw Data for Interior of Tumor-Implanted C3H/HeJ Mice with Heparin Injection

Sample No.	Days	PMNs	Lymph	Hist	Fib	Tumor
1	7	0	0	7	17	0
2	7	2	1	5	21	2
3	7	2	1	15	5	3
4	9	2	5	22	0	2
5	9	10	1	10	1	6
6	9	0	4	7	1	16
7	9	4	1	26	0	13
8	9	0	1	23	0	7
9	9	0	3	26	1	4
10	11	1	1	40	3	5
11	11	1	4	12	2	3
12	11	1	0	11	9	0
13	11	2	7	10	1	3
14	11	1	0	20	4	4
15	11	5	0	20	5	3
16	14	2	1	12	0	18
17	14	0	1	24	1	0
18	14	1	3	24	2	2

Table XXVII

EXPERIMENT III

Raw Data for Interior Tumor-Implanted C3H/HeJ Mice with Warfarin Injection

Sample No.	Days	PMNs	Lymph	Hist	Fib	Tumor
1	2	7	4	2	0	50
2	2	13	4	1	0	37
3	2	0	0	0	0	73
4	4	1	2	1	0	0
5	4	11	2	11	2	1
6	4	2	0	4	1	0

Table XXVIII

EXPERIMENT III

Raw Data for Interior of Tumor-Implanted C3H/HeJ Mice with Warfarin Injection

Sample No.	Days	PMNs	Lymph	Hist	Fib	Tumor
1	7	9	2	20	5	4
2	7	3	7	22	3	2
3	7	7	4	13	1	1
4	7	0	2	15	2	5
5	7	5	4	14	2	1
6	7	2	2	13	0	0
7	9	1	3	15	10	3
8	9	0	7	7	6	1
9	9	0	5	10	0	0
10	9	0	27	15	7	0
11	9	0	78	22	0	0
12	9	4	34	24	7	1
13	11	1	1	5	2	0
14	11	1	0	14	2	1
15	11	1	0	15	1	3
16	11	0	0	14	6	0
17	11	0	0	20	9	0
18	11	1	0	20	1	0
19	11	1	1	31	5	6
20	11	2	1	40	2	4
21	11	1	6	35	4	0

Table XXIX

EXPERIMENT III

Mean Values ± 1 Standard Error of the Mean

with Dunnett's-t applied for the Boundary of the

Tumor-Implanted C3H/HeJ Mice with and without Anticoagulants

Cell Type	Days	No. of Observations (S)	(H)	(W)	Saline Mean ± SEM	Heparin Mean ± SEM t=	Warfarin Mean ± SEM t=	Results p-value ct=
PMNs	1-4	12	12	6	8.2	6.4	10.2	p>0.05
					2.06	1.60	2.73	
						0.61	-0.70	2.34
	7-14	24	18	21	0.4	1.0	1.0	p>0.05
					0.12	0.31	0.24	
						-1.98	-1.83	2.27
Lymph	1-4	12	12	6	4.1	3.6	2.0	p>0.05
					0.71	0.96	0.93	
						0.41	1.66	2.34
	7-14	24	18	21	0.5	*2.4	*2.4	*p<0.05
					0.17	0.70	0.66	
						*-2.66	*-2.57	*2.27
Hist	1-4	12	12	6	7.2	7.1	5.7	p>0.05
					1.45	1.78	1.52	
						0.04	0.63	2.34
	7-14	24	18	21	6.5	9.3	9.1	p>0.05
					1.18	1.24	1.18	
						-1.61	-1.53	2.27
Fib	1-4	12	12	6	2.9	2.8	1.8	p>0.05
					1.29	0.88	0.91	
						0.10	0.68	2.34
	7-14	24	18	21	4.0	2.3	*1.2	*p<0.05
					1.05	0.66	0.45	
						1.87	*2.48	*2.27
Tumor	1-4	12	12	6	2.2	0.2	0.5	p>0.05
					1.06	0.11	0.34	
						1.87	1.56	2.34
	7-14	24	18	21	0.4	1.0	0.2	p>0.05
					0.16	0.29	0.12	
						-2.17	0.66	2.27

t= Dunnett's-t, ct= Critical-t, (S)= Saline, (H)= Heparin, (W)= Warfarin, *p<0.05= Significant Value with t value > ct. SEM= Standard Error of the Mean.

Table XXX

EXPERIMENT III

Mean Values ± 1 Standard Error of the Mean

with Dunnett's-t applied for the Interior of the

Tumor-Implanted C3H/HeJ Mice with and without Anticoagulants

Cell Type	Days	No. of Observations (S)	(H)	(W)	Saline Mean ± SEM	Heparin Mean ± SEM t=	Warfarin Mean ± SEM t=	Results p-value ct=
PMNs	1-4	12	12	6	2.5	3.8	5.7	p>0.05
					0.92	0.97	2.25	
						-0.74	-1.87	2.34
	7-14	24	18	21	1.7	1.9	1.9	p>0.05
					0.38	0.58	0.54	
						-0.26	-0.21	2.27
Lymph	1-4	12	12	6	2.3	1.7	2.0	p>0.05
					0.43	0.38	0.73	
						0.88	0.38	2.34
	7-14	24	18	21	2.4	1.9	8.8	p>0.05
					0.35	0.47	3.96	
						0.16	-1.95	2.27
Hist	1-4	12	12	6	5.0	8.2	3.2	p>0.05
					1.26	1.39	1.66	
						-1.57	0.91	2.34
	7-14	24	18	21	14.6	17.4	18.3	p>0.05
					1.57	2.14	1.90	
						-1.07	-1.39	2.27
Fib	1-4	12	12	6	5.0	2.5	0.5	p>0.05
					2.29	0.74	0.34	
						1.05	1.88	2.34
	7-14	24	18	21	5.2	4.1	3.6	p>0.05
					0.82	1.40	0.66	
						0.82	1.18	2.27
Tumor	1-4	12	12	6	4.9	6.2	*26.8	*p<0.01
					1.84	1.30	12.75	
						-0.20	*-3.42	*3.04
	7-14	24	18	21	3.4	5.1	1.5	p>0.05
					0.71	1.25	0.41	
						-1.43	1.65	2.27

t= Dunnett's-t, ct= Critical-t, (S)= Saline, (H)= Heparin, (W)= Warfarin, *p<0.01= Significant Value with t value > ct. SEM= Standard Error of the Mean.

DISCUSSION AND ANALYSIS

In normal transplant rejection, histoincompatible cells from the donor would activate the immune response in the recipient. This would activate all aspects of the defense system (macrophages, B lymphocytes, T lymphocytes, and complement) of which the macrophage and the T lymphocyte would be the most effective. Therefore, in human organ transplant recipients every effort would be used to suppress the immune response so that the transplant graft tissue could survive. Studies of privileged tissues, graft sites, and antigenic tolerance have been important in developing methods to escape the graft rejection phenomenon(20).

The tumor cells used in each of the three experiments have some antigenic similarity to host cells but are not antigenically identical to them. For this reason it is not clear if the host's immune response is incompletely activated and fails to reject the tumor or if factors released by the tumor (angiogenesis factors, vascular permeability factors, enhancing factors, or blocking factors) enhance rather than inhibit tumor growth(20).

Many but not all animal tumor cells have tumor specific antigen on their surfaces. Most of these antigens are immunogenic and produce either a humoral or cell-mediated immune response. The cell-mediated (T lymphocyte) response

may be neutralized by serum-blocking factors. It is possible that tumors fail to be recognized by the immune system or cause only a weak response(13).

The goal of Experiment I was to become familiar with the parenchyma and stroma of C3H/HeJ mouse mammary tumors both spontaneous and passaged in order to evaluate morphologic responses to experimental procedures. Familiarity with the parenchyma and stroma was achieved by reviewing slides of tumor samples from each spontaneous tumor mouse under light microscopy at 400 power. These slides were then compared to slides of tumor samples from corresponding first and second transplant tumor mice, respectively. Slides were reviewed first for acinar or glandular arrangement of the mouse mammary tumor as compared to the normal mouse mammary glands; second for the presence or absence of intraluminal secretions (the presence of secretions indicating a more normal functional mammary tissue and the absence of secretion indicating a more diffuse, less differentiated, and more neoplastic development). Third, the tumor cells were observed for presence or absence of cytoplasm around their nuclei. The presence of cytoplasm was associated with acinar or glandular arrangement. The absence of cytoplasm was noted in the presence of increased cellularity.

As observed in Table IV, some spontaneous mouse mammary tumors were more anaplastic than others. ST4 was the most anaplastic with a cellular density comparable to

that seen after the second passage in the other three groups.

Since tumors become more anaplastic with each transplantation(11) it would appear that ST4 had become very anaplastic spontaneously, resulting possibly in a greater concentration of cells prior to implant. This phenomenon cannot be explained by rapid tumor growth alone, but is related to increased malignancy referred to as tumor progression. The phenomenon is also observed in an advanced tumor in a host over time. In tumor progression the progeny of cancer cells exhibit increasingly malignant characteristics, whereas, the progeny of healthy cells are usually faithful copies of their parent(11). As might be expected with an increased tumor concentration, necrosis was observed in TP4-1 and TP4-2 (sequential transplants 1 and 2 of spontaneous tumor number 4) indicating inadequate blood supply and cell death.

In contrast, ST1 (Table IV) had a very low cell density with no tumor cells noted until after two transplants (TP1-1 and TP1-2), and with tumor cells noted only in TP1-2. Since approximately 10 mg of tumor was implanted into each mouse, it would seem clear that part of the 10 mg implanted from ST1 was fat or muscle with a very small number of tumor cells present. The general absence of tumor also explains the absence of glandular arrangement, secretions and cytoplasm, since there would be a decreased amount of mammary tissue transplanted as well.

Spontaneous tumor number two (ST2) had a glandular arrangement present with secretions and cytoplasm. The morphology was very similar to normal mammary tissue and suggests a less malignant tumor. The transplants (TP2-1 and TP2-2) seem to confirm the initial observation since the glandular arrangement decreased but was still present after the second transplant (TP2-2) and the cytoplasm remained present, but associated only with acinar arrangement. One indication of increased malignancy or tumor progression was the absence of secretions in TP2-1 and TP2-2 where it was present in ST2.

ST3 appeared to be more anaplastic than ST2 but less than ST4. In the original sample, there was some glandular arrangement noted, with secretions and cytoplasm present, but with one transplant (TP3-1) all glandular arrangement, secretions and cytoplasm disappeared indicating a relatively more malignant tumor. TP3-2 confirmed the pattern of change with a densely increased cellularity.

In summary the order of malignancy proceedes from ST4 > ST3 > ST2 > ST1. Progressive tumor developement is indicated by a decrease or absence of glandular arrangement, secretions and cytoplasm, with an increase in cellularity. It should be noted that not all tumors are equally malignant but all progress toward anaplasticity.

Experiment II was expected to demonstrate a wide range of leukocytic activity with early inflammatory response to the sham operation and liver implantation. An increase in

neutrophil (PMN) and macrophage activity was expected due to possible inflammation caused by dead liver cells. As seen in (Table XV) there was a stastically significant ($P<0.05$) increase in boundary PMNs between days 1 and 4 for the tumor-implanted mice, when compared to the sham operated mice. There was also an increase in the boundary PMNs between days 1 and 4 for the liver-implanted mice, when compared to the sham operated mice. However, the change was not statistically significant by day 4 with a resolution of the inflammation and a reduction of PMNs in all three groups by day 8. These results seem to indicate a normal immune response by PMNs to both liver and tumor implants. These data also indicate that the tumor was at least as exposed to PMN attack as the liver, since a significantly larger mean value of PMNs was observed for the tumor-implanted group than for the liver-implanted group. This increase in PMNs is probably related to the phagocytic activity of the PMNs(20) and the antigenic exposure on the tumor cells. It should also be noted from Table XVI that early, day 1 through day 4, there was less PMN penetration into the tumor when compared to liver. This difference can be explained in terms of phagocytosis of dead liver cells compared to viable tumor cells or in terms of the tumor preventing penetration by a protective mechanism(12).

An increased day 1 through 4 boundary lymphocytic response was observed in both liver- and tumor-implanted groups when compared to the sham operated group. Values for

both liver- and tumor-implanted boundary lymphocytes were statistically increased ($P<0.01$). These increases may reflect the presence of surface antigens on the tumor or a lack of blocking ability by the tumor, and a response to decaying necrotic tissue from the liver implant. Table XVI demonstrates a late day 8 increase ($P<0.05$) in the number of lymphocytes penetrating the tumor implant when compared to the number of lymphocytes penetrating the liver implant. This increase may be related to the death of the liver implant and the continued viability of the tumor implant.

The increased ($P<0.05$) number of day 1 through 4 boundary histiocytes observed in the liver-implanted group when compared to the sham operated group further supports the possibility of immune response to decaying necrotic liver cells. Table XVI further supports this observation with a decrease ($P<0.05$) in the day 1 through 4 number of histiocytes penetrating the tumor implant when compared to the liver implant. This could indicate histiocytic response to necrotic tissue in the liver or a decreased response to the tumor implant due to its protective mechanism, or a combination of the two. Since histiocytes are nonspecific in inflammatory response, an increase was expected in all three groups due to the implantation procedure alone. The late decrease in histocytic response to the tumor implant when compared to the liver implant in Table XVI and in Table XV, although not statistically significant, approaches the inflammatory response observed in the sham operated group in

Table XV. This would seem to indicate that the tumor is protected and that the necrotic liver implant is being exposed to phagocytosis.

The increased lymphocytic infiltrate coupled with the decreased histocytic influx into the tumor's interior suggest that despite an immune presence, amplification of responses (i.e., increased histiocytes) are quelled. This follows Dvorak's finding of MIF release by tumor cells(12).

The reason for a significant decrease ($P<0.01$) in the number of fibroblasts observed from day 1 through 4 in both the liver- and tumor-implanted groups when compared to the sham operated group is unclear, but may be related to stretching of the tissue caused by the implants and by edema. Later, with decreased edema, as the inflammation resolves and repair gets underway a relative increase in the number of fibroblasts would be expected in the liver- and tumor-implanted groups. This increase was observed (Table XV) in both the liver- and tumor-implanted groups, although not statistically significant.

One theory of tumor adaptability is that tumors release factors such as: 1) vascular permeability factor which causes the blood vessels to become leaky; 2) procoagulation factor which can cause a clot to form around the tumor (a fibrin cocoon); and 3) plasmin (proteolytic enzyme) activator factor which breaks down the fibrin cocoon. The fibrin cocoon could cause a decrease in visibility of the tumor to the immune system by covering up the surface

antigen. There is also some evidence that the fibrin cocoon is related to angiogenesis possibly stimulating the development of new blood vessel formation(21).

Tumor angiogenesis factor is a diffusable chemical substance which is released from tumor cells. It has the capacity to stimulate nearby blood vessels to send out new capillaries that grow toward the colony of tumor cells and finally penetrate it. Rapid tumor growth then follows with fresh nutrients poured in and wastes speedily removed(11).

Given these findings and others from Santer(17) the tumor implants of Experiments I and II were expected to cause angiogenesis with rapid growth, with an increased null cell population, a decreased number of T lymphocytes and an unchanged number of B lymphocytes . The results of Experiment I (Table IV) did indicate a more anaplastic growth pattern with first and second passage of the tumor which was also demonstrated by Folkman(11). However, there was no decrease, but an increase in total overall lymphocyte response to both liver-implanted and tumor-implanted C3H/HeJ mice in Experiment II (Table XV and Table XVI), when compared to the sham operated group. Again, these increases may reflect the exposure of surface antigens on the tumor implant or a lack of blocking ability by the tumor, and a response to decaying necrotic tissue from the liver implant.

Experiment III was designed to study the effects of the fibrin cocoon and to analyze changes which might occur if

the fibrin protection was removed by the use of heparin or warfarin. Both heparin and warfarin act by different mechanisms to inhibit or prevent fibrin cocoon formation. If the fibrin protection was necessary for angiogenesis(21) a reduced growth of tumor in the mice on heparin or warfarin versus the control mice on saline could be anticipated, with an increased immune activity if the fibrin had blocked recognition in those mice on anticoagulants.

Based on the studies by Folkman(11) and Santer(17) early increased PMN and macrophage activity should be seen in the saline control group, with inflammation from the implantation procedure. Later there should have been an increase in angiogenesis, a rapid growth of the tumor, and an increase in null cells, with a decrease in T lymphocytes and no change in B lymphocytes.

In the heparin and warfarin treated tumor mice early increases in PMN and macrophages would be expected if an inflammatory response was caused by the implantation procedure. However, a decrease in angiogenesis with decreased growth of the tumor would occur if the fibrin cocoon was important in tumor protection and development. An increase in null cell activity associated with transplant tumors and an increase in T lymphocytes and B lymphocytes has been observed(17). With decreased tumor protection, normal immune surveillance should produce these increases. Antigens should be exposed on the surface of the tumor cells and B lymphocytes should have been

stimulated to produce antibodies as a late development.

As seen in Table XXIX there was a general increase in the number of PMNs from day 1 through 4, probably related to the inflammation caused by the implantation procedure used to implant the tumors. By day 7 through 14 there was a decreased number of PMNs at the boundary of the saline injected group when compared to the heparin and warfarin injected groups. The results are not statistically significant but a trend toward decreased protection of the tumor in the heparin and warfarin groups seems possible with a resolution of the inflammation resulting in an overall decrease in boundary PMNs. Table XXX further supports this assumption in that days 1 through 4 show a greater number of PMNs penetrating to the interior of the tumor in the heparin and warfarin groups when compared to the saline group, but again, the numbers are not statistically significant. These trends seem to indicate an increased immune response in the heparin and warfarin groups when compared to the saline group. This could be explained by a decrease in the protective fibrin-gel hypothesized by Dvorak(12).

As indicated in the studies by Folkman(11) and Santer(17) there was a late increase (Table XXIX) in the number of lymphocytes in the heparin ($P<0.05$) and warfarin ($P<0.05$) groups when compared to the saline group. This late increase in lymphocytes in both the heparin and warfarin groups indicates less surface protection of the tumor from immune surveillance. Table XXX projects a similar trend

when comparing the late warfarin group to the saline group but the increase is not statistically significant. There is an early (day 1 through 4) and late (day 7 through 14) increase in the number of lymphocytes in the interior of the saline group when compared to the heparin group, but the increase is not significant and the reason is unclear.

Activation of macrophages probably occurs when an antigen activator stimulates thymus-derived lymphocytes that may produce histologically active molecules responsible for modification of macrophage function. Activated macrophages spare contact-inhibited cells regardless of the antigen composition but destroy non-contact-inhibited cells regardless of the antigen composition. They probably respond to cell surface changes associated with the expression of the neoplastic phenotype. This is considered a nonimmunologic response. Immune macrophages response is different and is specific for target cells(13).

There seems to be a trend toward late (day 7 through 14) histiocyte or macrophage activity in both the heparin and warfarin groups when compared to the saline group in Table XXIX. This same trend is observed in Table XXX with increased penetration into the tumor by histiocytes in the heparin and warfarin groups. Although, the numbers are not significantly increased, there seems to be a trend toward increased macrophage activity in the anticoagulated groups at the boundary and in the interior,indicating less protection, when compared to the saline group.

There is a decreased ($P<0.05$) number of fibroblasts in the day 7 through 14 warfarin group (Table XXIX) when compared to the saline group. This could be the result of decreased clot formation, due to the warfarin, leading to a decrease in the number of fibroblasts and decreased wound healing or repair. It should also be observed from day 1 through 4 that there is a larger number ($P<0.01$) of tumor cells (Table XXX) in the warfarin group than in the saline group. It is interesting to note that with early day 1 through 4 decrease in fibroblasts that there is an incresae ($P<0.01$) in the number of tumor cells. This may be due to the decreased containment of the tumor with decreased fibroblasts. However, the reason for an increased ($P<0.01$) number of tumor cells in the warfarin group when compared to the saline group is unclear.

SUMMARY

Three separate experiments were conducted to determine the nature of leukocytic response to mouse mammary tumor implants in C3H/HeJ mice with and without anticoagulants.

In Experiment I the investigator became familiar with the parenchyma and stroma of spontaneous and passaged C3H/HeJ mouse mammary tumors in order to evaluate morphologic responses to experimental procedures. A light microscope was used to look at the morphology of spontaneous and passaged C3H/HeJ mouse mammary tumors. Four C3H/HeJ spontaneous tumor mice were used in the experiment. A single segment of approximately 10 mg of tumor from each mouse was implanted into two virgin female C3H/HeJ mice. Three weeks after the implantation the two mice (first passage) were sacrificed. A segment of tumor from one mouse was implanted into a normal mouse and a segment of the second tumor was implanted into a second normal mouse. Three weeks later the two mice (second passage) were sacrificed.

The following conclusions appear justified from Experiment I:

(i) Acinar or glandular arrangement of the mouse mammary tumor decreases as the tumor becomes more metastatic.

(ii) Secretions associated with a normal function-

al mammary tissue decreases and becomes absent in advanced spontaneous mouse mammary tumors and with first and second passage as the tumor becomes more anaplastic.

(iii) Cytoplasm around the nuclei of the tumor cells decreases with increasing numbers of cells. The cytoplasm becomes absent in the presence of dense cellularity seen with advanced spontaneous tumors and when the tumor becomes more anaplastic.

Experiment II characterized the leukocytic response to implanted C3H/HeJ spontaneous tumor segments by contrasting that response with those of syngeneic mice with implants of normal (liver) tissue and mice that were sham operated upon. Three groups of 12 mice (Table II) were used to conduct the experiment. A female donor C3H/HeJ mouse with a spontaneous mammary tumor and a normal mouse were sacrificed to obtain tumor and liver for implanting the two groups of twelve mice. Each of the three groups were observed for changes in polymorphonuclear cells (PMNs), lymphocytes (Lymphs), histiocytes (Hist), and fibroblasts (Fib) around the boundary of the implant site and, where applicable, in the interior of the liver or tumor.

The following conclusions appear justified in Experiment II:

(i) There was a greater ($P<0.05$) immune response (Table XV) by boundary PMNs between days 1

and 4 in the tumor-implanted mice when compared to the sham operated mice. This indicates that the tumor implant was at least as exposed to attack by PMNs as the liver implant, since a significantly larger mean value of PMNs was observed for the tumor-implanted group than for the liver-implanted group.

(ii) There were approximately 66% fewer PMNs penetrating to the interior of the tumor implant than was observed penetrating the interior of the liver implant. This may indicate interior protection by the tumor by some mechanism different from the liver, however the results were not statistically significant.

(iii) The lymphocyte response (Table XV) was significantly ($P<0.01$) increased in both the liver- and tumor-implanted groups when compared to the sham operated group. These increases may reflect the presence of surface antigens on the tumor or a lack of blocking ability by the tumor, and a response to decaying necrotic tissue from the liver implant.

(iv) Table XVI demonstrates a late day 8 increase ($P<0.05$) in the number of lymphocytes

penetrating the tumor implant when compared to the number of lymphocytes penetrating the liver implant. This increase may be related to the death of the liver implant and the continued viability of the tumor implant.

(v) The boundary histiocyte response between days 1 and 4 was greater ($P<0.05$) in the liver implant group when compared to the sham operated group. This probably indicates an immune response to decaying necrotic liver cells.

(vi) The interior histiocyte response between days 1 and 4 was significantly less ($P<0.05$) in the tumor-implanted group when compared to the liver-implanted group probably related to protective mechanisms and to the viability of the tumor compared to the decaying liver tissue.

(vii) The fibroblasts were significantly ($P<0.01$) less around the tumor and liver implants. This reduction in number was probably related to edema and stretching produced by the implants with insufficient time by day 4 for repair.

Experiment III described changes in leukocytic response to tumor implants in control (saline injected) tumor-bearing mice versus heparin or warfarin injected tumor-bearing mice.

Three groups of 12 mice were implanted with tumors. One group was injected over 14 days with saline, a second group with heparin and the third group with warfarin.

The following conclusions appear justified from Experiment III:

(i) There was a general increase in the number of PMNs from day 1 through 4, probably related to the inflammation caused by the implantation procedure used to implant the tumors.

(ii) There was a late increase ($P<0.05$) in the number of lymphocytes in both the heparin and warfarin groups when compared to the saline group (Table XXIX). This late increase in lymphocytes in both the heparin and warfarin groups indicates less surface protection of the tumor from immune surveillance.

(iii) The number of fibroblasts was significantly ($P<0.05$) reduced at the boundary of the warfarin group between days 7 and 14 (Table XXIX). This could be the result of decreased clot formation due to the warfarin leading to a decrease in the number of fibroblasts and decreased wound healing and repair.

(iv) There was a significant increase ($P<0.01$) in the number of tumor cells (Table XXX) in the

warfarin group when compared to the saline group. The reason for this increase in unclear. The increase may have been related to the number of viable tumor cells implanted, or to the blood supply to the tumor from the host, but further research seems indicated.

The nature of leukocytic response to mouse mammary tumor implants in C3H/HeJ mice with and without anticoagulation seems to be varied.

The author found significant increases in the number of neutrophils (PMNs) ($P<0.05$), lymphocytes ($P<0.01$), and fibrocytes ($P<0.01$) in the tumor-implanted group when compared to the sham operated group of C3H/HeJ mice in Experiment II (Table XV). There was also an increase ($P<0.05$) in the number of lymphocytes on day 8 (Table XVI) in the tumor-implanted group when compared to the liver-implanted group of C3H/HeJ mice. All of these responses may be indicative of a normal host immune response to an inflammatory procedure (i.e., the implantation procedure used to implant the liver and tumor) in non-coagulated C3H/HeJ mice.

Experiment III (Table XXIX), which has tumor implants in all three groups, shows a significantly increased number of lymphocytes ($P<0.05$) from day 7 through 14 in the heparin and warfarin injected groups when compared to the saline injected group of C3H/HeJ mice. There was also a decreased

($P<0.05$) number of fibroblasts in the day 7 through 14 warfarin injected group when compared to the saline injected group. Table XXX shows a day 1 through 4 increase ($P<0.01$) in the number of tumor cells in the warfarin injected group when compared to the saline injected group. All of this information seems to indicate that the tumor is protected in the saline injected group and that the tumor is exposed in the anticoagulated (heparin and warfarin) groups, with the warfarin group being more exposed than the heparin group. The decreased number of fibrocytes in the warfarin group could represent less confinement of the tumor or greater exposure of the tumor.

The levels of significance found throughout Experiments II and III indicate that this new area of study deserves further research with the hope of improved response, in a clinical sense, in the patient with metastatic cancer.

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